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Bax and Bcl-x_s are induced at the onset of apoptosis in involuting mammary epithelial cells

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Abstract

Mammary gland involution is a physiological process in which the entire organ is remodeled through the process of apoptosis. Apoptosis of secretory alveolar cells is initiated at the time of weaning, followed by the collapse and disappearance of the entire lobulo-alveolar compartment. While apoptotic figures were rare in mammary epithelium of lactating mice, their number increased after weaning and reached a maximum on day 3 of involution. Active cell death continued until day 5 after weaning and only little parenchyma remained on day 8, when remodeling of the gland was completed. Bax mRNA levels increased during the first day of involution independent of the presence or absence of p53. Bax protein was detected in an increasing number of cells after weaning, peaking at day 3 and decreasing thereafter. Low levels of bcl-x mRNA and protein were present during lactation, followed by a sharp increase during the first 2 days of involution. The bcl-x_S splice variant of bcl-x can promote cell death, and bcl-x_L has a protective function in cell culture. The ratio of bcl-x_S versus bcl-x_L remained stable in the virgin, pregnant and lactating gland. However, during the first 2 days of involution, bcl-x_S expression increased six-fold more than bcl-x_L. To further evaluate the role of Bcl-x_S which was less abundant in the mammary cells than Bcl-x_L, cotransfection studies were performed in cell culture. They confirmed that Bcl-x_S protein can facilitate apoptosis even when Bcl-x_L is present in excess. These findings point to a significant role for Bax and Bcl-x_S in the regulation of apoptosis of secretory alveolar cells during involution.

Keywords: Bax; Bcl-x_S; Apoptosis; Mammary gland involution

1. Introduction

A hallmark of the mammary gland is its regenerative capacity. In virgin mice, an organized system of ducts penetrates the mammary fat pad. Extensive ductal and lobulo-alveolar development mediated by lactogenic hormones and local growth regulators such as TGF-β1 (for references see Kordon et al., 1995) commences with each pregnancy and is completed at the onset of lactation. Development and functional differentiation of alveoli during pregnancy leads to the sequential expression of milk protein genes (Robinson et al., 1995), followed by milk secretion and lactation. After weaning of the pups the mammary gland involutes and proceeds through a rapid remodeling process which reduces the tissue to a state that resembles the mature virgin gland (Helminen and Ericsson, 1968; Walker et al., 1989). The complete loss of secretory alveolar cells, combined with the proteolytic degradation of extracellular matrix, leads to the collapse and disappearance of lobuloalveolar structures. The majority of cells that die during involution undergo apoptosis (Walker et al., 1989; Strange et al., 1992; Guenette et al., 1994). They show cellular condensation and nuclear fragmentation and are eventually engulfed by macrophages and neighboring epithelial cells.

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The transition from lactation to involution is accompanied by declining levels of systemic lactogenic hormones and the mammary gland transcription factor (MGF or Stat5), the rapid inactivation of milk protein genes, mild ischemia, and increasing intraluminal pressure, all of which may be involved in the induction of cell death and tissue remodeling (Silver, 1956; Ota et al., 1962; Richards and Benson, 1971; Schmitt-Ney et al., 1992). At the same time the expression of genes possibly involved in tissue remodeling is induced (Dickson and Warburton, 1992; Lefebvre et al., 1992; Strange et al., 1992; Guenette et al., 1994; Marti et al., 1994; Boudreau et al., 1995). However, the trigger that decides whether individual epithelial cells will survive or undergo apoptosis is not known.

Different cell types may utilize different pathways to undergo apoptosis depending on their developmental needs. For example, the lens and the choroid plexus develop just once, whereas mammary gland development is repeated with each pregnancy. While neither the lens nor the choroid plexus undergo extensive apoptosis during a lifespan, the entire alveolar compartment of the mammary gland is eliminated after weaning and the tissue is completely remodeled. This occurrence of fundamentally different developmental processes raises the question whether apoptosis pathways are shared between different cell types, or whether distinct pathways are used. Apoptosis in the choroid plexus (Symonds et al., 1994) and the lens during early stages of development (Pan and Griep, 1996) is mediated through p53-dependent pathways. Only recently the concept has emerged that, depending on the cell type or the physiological condition, either p53-dependent or -independent pathways can be activated (Pan and Griep, 1996a; Li et al., 1996a and references therein).

Bcl-2 promotes cell survival and is the prototype of a family of genes that regulate apoptosis (Hockenbery et al., 1990; Oltvai et al., 1993; Motoyama et al., 1995; Takayama et al., 1995; Yang et al., 1995). Deletion of the bcl-2 gene from the genome had no overt effects on embryogenesis (Veis et al., 1993). Their mammary glands develop normally, indicating that Bcl-2 does not play a dominant role in mammary gland remodeling. Bax shares 21% amino acid sequence homology with Bcl-2 (Oltvai et al., 1993) and has been shown to induce cell death in cell culture. Bax-deficient mice (Knudson et al., 1995) display hyperplasia in their lymphoid system, supporting a role for Bax as a death-facilitating protein. The same animals, however, show enhanced cell death in their reproductive organs and exhibit aberrant mammary gland development (Korsmeyer, personal communication), creating a more complex picture of different functions Bax may fulfill in different cellular contexts. Two bcl-x mRNA splice variants exist, a long form, bcl-x_L, encoding a protein that protects cells from death, and a short form, bcl-x_S, that encodes a protein-promoting death (Boise et al., 1993). Mice lacking functional Bcl-x die during embryogenesis and massive cell death can be observed in the nervous system indicating a critical role for bcl-x in the developing organism (Motoyama et al., 1995).

Based on our study of SV40 T-antigen transgenic mice in which apoptosis can be induced precociously (Li et al., 1996b), it appears that both bcl-x and bax could be critical components of alveolar cell death and mammary gland remodeling during normal involution. To test this hypothesis we performed a detailed expression analysis of bax and bcl-x during normal involution of mammary tissue.

2. Results

2.1. Apoptosis of mammary epithelial cells

To investigate morphological and molecular changes during early stages of involution, pups were removed from their mothers at day 10 of lactation. The Tunel assay was used to identify dying cells containing fragmented DNA. During lactation, mammary tissue consisted of secretory alveoli and ducts with epithelial and myoepithelial cells embedded in fatty tissue. Only a few epithelial cells with fragmented DNA were detected (Fig. 1A). Twelve hours and 2 days after weaning, increasing numbers of cells underwent apoptosis (Fig. 1B,C); some of them were shed into the alveolar lumina. The peak of apoptotic cell death was seen at day 3 after weaning, when remodeling of the mammary epithelium was most pronounced (Fig. 1D). Lobulo-alveolar structures became smaller, leading to an overall morphological change. On day 5 of involution alveoli were further reduced in number and size and apoptotic figures became less frequent (Fig. 1E). At day 8, regression of the gland was completed with few lobules and ducts remaining in the fatty tissue (Fig. 1F).

2.2. bcl-x and bax RNA levels in mammary alveolar cells increase during involution

The histological observations are indicative of massive cell loss by apoptosis during the first week after weaning. Underlying changes in the expression of the bax and bcl-x genes during involution were studied on Northern blots (Fig. 2), in situ hybridization (Fig. 3), Western blots (Fig. 4) and immunohistochemistry (Fig. 5). Low levels of bcl-x mRNA were detected during lactation (Fig. 2A). Up to 12 h after weaning bcl-x mRNA expression was low, but increased after 24 h and remained high throughout involution. Similar to bcl-x, bax mRNA levels were low during lactation. Within 6 h after weaning, expression increased and stayed high until day 3 of involution. After day 3 the amount of bax mRNA declined (Fig. 2B). In situ mRNA hybridization of bcl-x and bax showed weak and homogeneous labeling of epithelial cells during lactation (Fig. 3A,D). One day after weaning label intensity had increased (Fig. 3B,E), slowly leveling out later in

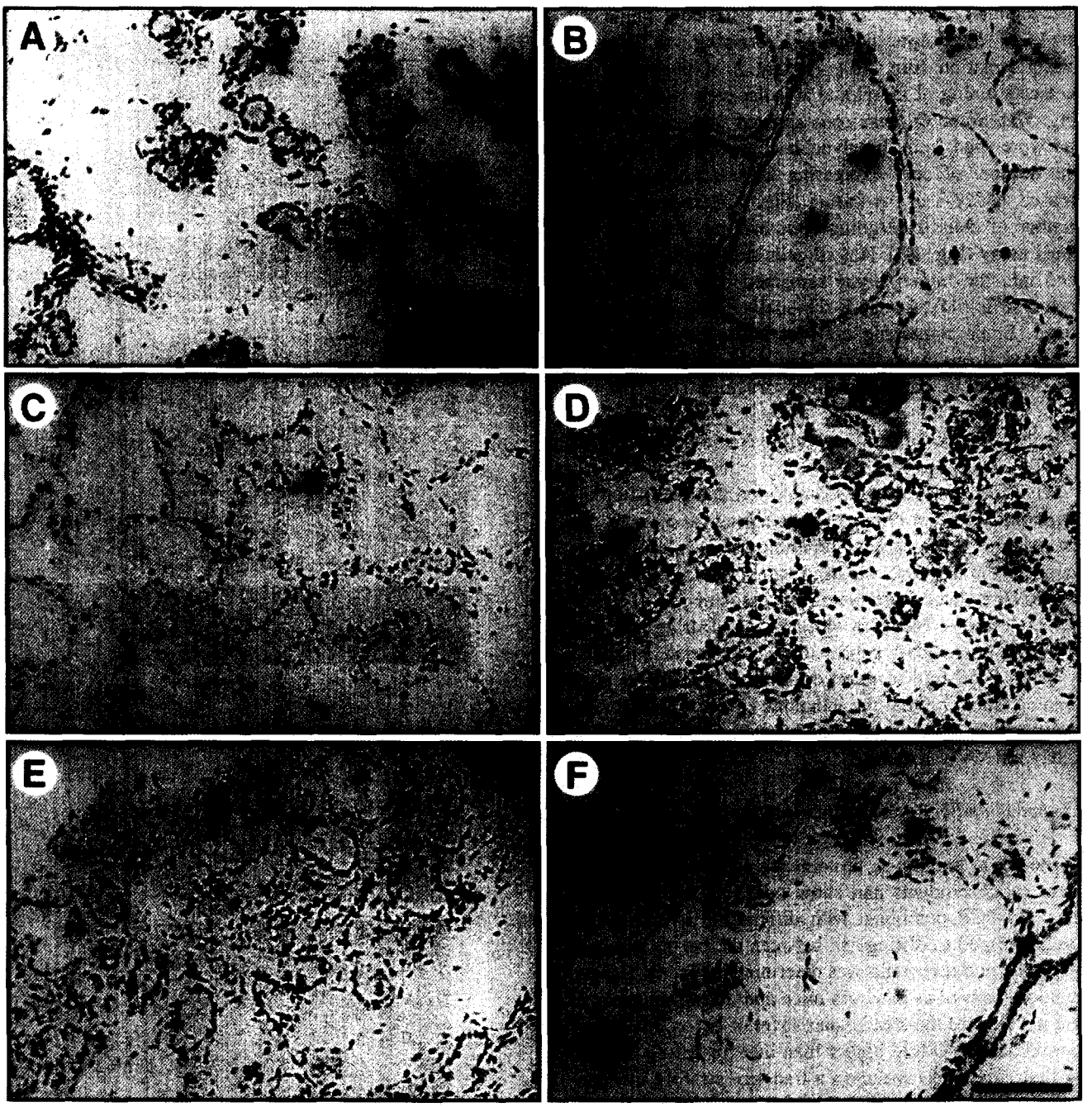

Fig. 1. Tunel staining of involuting mammary glands. (A) At day 10 of lactation, few apoptotic cells are observed (arrow). (B) Twelve hours after weaning apoptotic cells are located in the epithelium (arrow) and shed into the lumina. (C) Involution day 2. (D) Massive apoptosis (arrows) occurs 3 days after weaning. (E) The number of apoptotic cells (arrow) is reduced after 5 days. (F) After 8 days the apoptotic process is completed. lu, lumen; du, duct. Bar is 100 μm.

involution, thereby confirming the Northern blot analysis (Fig. 3C,F).

2.3. *Bax protein localizes to apoptotic mammary alveolar cells*

Western blots and immunohistochemistry were performed to evaluate the presence of Bcl-x and Bax protein. Bax has a molecular weight of 21 kDa and was specifically recognized by an anti-bax peptide antibody (Fig. 4). It was present throughout involution and its concentration increased late in involution. Bcl-x_L, which has a size of 30 kDa, was present at low levels during lactation, followed by an increase upon weaning (Fig. 4). The decision whether or not a cell will undergo apoptosis may depend on the relative levels of each protein in an individual cell. Since Western blots can determine only the overall levels of bax or bcl-x within the tissue, we performed immuno-

histochemistry to evaluate bax expression on a single cell level (Fig. 5). Homogeneous weak staining was observed during lactation (Fig. 5A). At day 2 of involution increased staining of individual alveolar cells was observed (Fig. 5B). Some of these cells showed fragmented nuclei and were shed from the alveolar context into the lumen. The number of cells expressing high levels of bax increased at day 3 of involution (Fig. 5C), as seen for the number of cells undergoing apoptosis as judged by the Tunel assay (Fig. 1D). Not all cells staining for bax were shed into the lumen, some remained part of collapsing alveoli (Fig. 5D). At day 8 remodeling was almost completed. Some remaining cells of collapsed alveoli still exhibited strong bax staining, but no significant staining was observed in remodeled ductal structures (Fig. 5F).

2.4. *Bax induction during involution is p53-independent*

Miyashita and Reed (1995) reported that p53 is a transcriptional activator of bax in human lung cancer cells. To test whether p53 causes the induction of bax during mammary involution, we used mice lacking functional p53. RNA isolated from mammary tissue of p53 –/– mice was subjected to Northern blot analysis. During lactation only little bax mRNA expression was detected, followed by a 10- to 50-fold increase during the first 3 days after weaning, showing that the induction of bax expression is unchanged in the absence of p53. At day 10 of involution no bax mRNA was detected.

2.5. *bcl-x_L and bcl-x_S are differentially regulated*

The bcl-x_L and bcl-x_S proteins are encoded by two different splice products and show opposing cellular functions. RT-PCR combined with differential hybridization was performed to distinguish between the bcl-x_L and bcl-x_S mRNA and their ratio was determined (Fig. 6). During lactation (as well as in virgin mice and during pregnancy, data not shown) the steady state level of bcl-x_L RNA was approximately 40-fold higher than that for bcl-x_S. Within the first 2 days of involution a transient six-fold increase in the ratio of bcl-x_S to bcl-x_L was observed. The relative increase of bcl-x_S was observed at 12 h after weaning, reached a maximum after 48 h, and decreased thereafter (Fig. 6).

2.6. *The presence of bcl-x_S leads to loss of cell viability*

To test whether a relative increase of Bcl-x_S over Bcl-x_L can cause cell death, we transfected a murine IL-3-dependent pro B-cell line with an expression vector encoding bcl-x_L or with expression vectors encoding both the long and short form. Apoptosis was induced by treatment with etoposide or IL-3 withdrawal. In vector transfected control cultures 98% or 100% of the cells underwent apoptosis after 4 days of etoposide treatment or IL-3

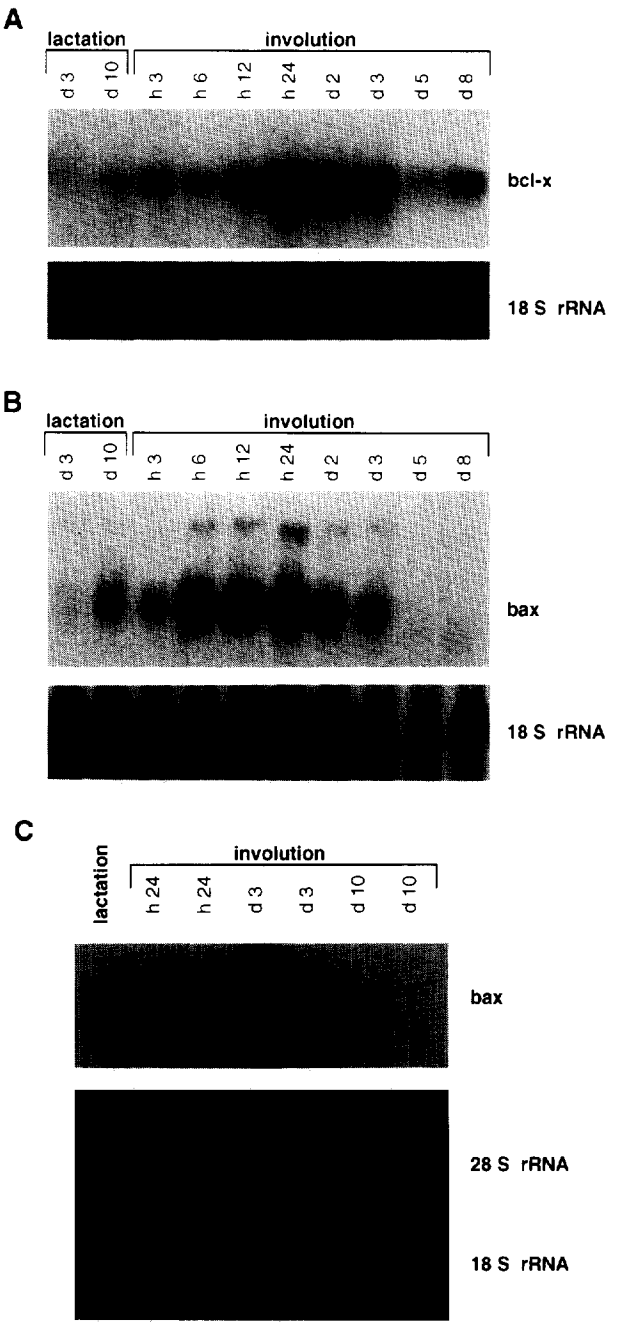

Fig. 2. Northern blot analysis of (A) bcl-x and (B,C) bax in (A,B) wildtype and (C) p53–/– mice. (A,B) Methylene blue or (C) ethidium-bromide stained 18 S rRNA was used as loading standard.

withdrawal, respectively. Expression of Bcl-x_L alone resulted in enhanced cell viability and only 27% of the cells died. Coexpression of Bcl-x_L and Bcl-x_S resulted in an inhibition of the protective effect of Bcl-x_L. In these cultures 80% of the cells died by apoptosis after 4 days of etoposide treatment (Fig. 7A) and 53% of the cells died after IL-3 withdrawal (Fig. 7B). Fig. 7C shows a Western blot analysis of Bcl-x protein levels in the different cell lines. The ratio of Bcl-x_L versus Bcl-x_S in cotransfected cells was 3:1.

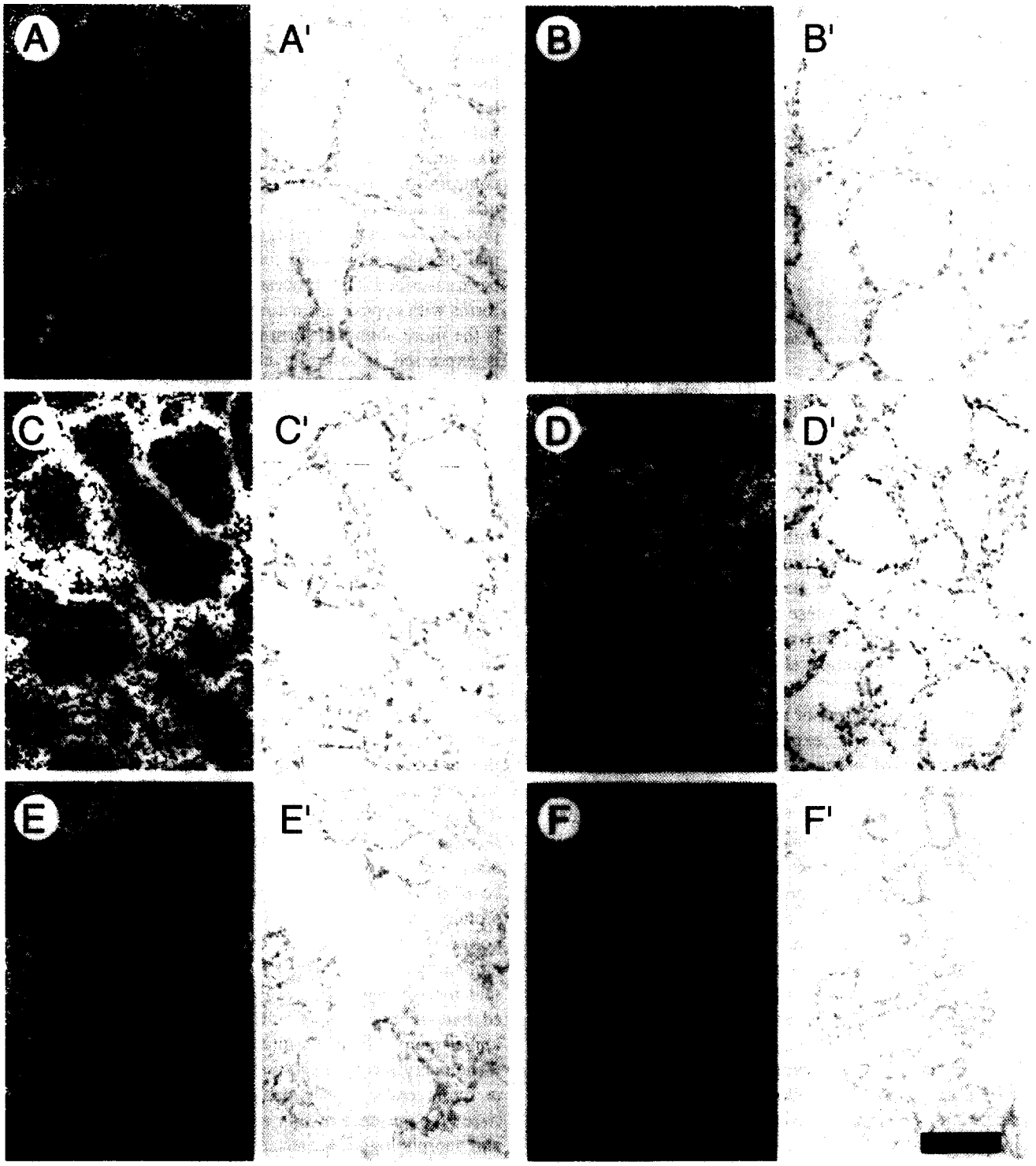

Fig. 3. Localization of (A,C,E) bcl-x and (B,D,F) bax mRNA in involuting mammary glands by in situ hybridization. (A,B) Day 10 of lactation; (C,D) day 1 and (E,F) day 3 of involution. Identical sections are shown in (A–F) darkfield and (A′–F′) brightfield view. Bar is 50 μm.

3. Discussion

Genes involved in apoptosis have been studied in cell culture (Hockenbery et al., 1990; Boise et al., 1993; Oltvai et al., 1993; Yonish-Rouach et al., 1993; Miyashita and Reed, 1995), in transgenic mice (Li et al., 1994) and in mice devoid of these genes (Donehower et al., 1992; Veis et al., 1993; Motoyama et al., 1995). However, at this point it is not clear which proteins mediate apoptosis during mammary gland remodeling. We now show that the expression pattern of two apoptosis genes, bax and bcl-x, parallels the timecourse of apoptosis during mam-

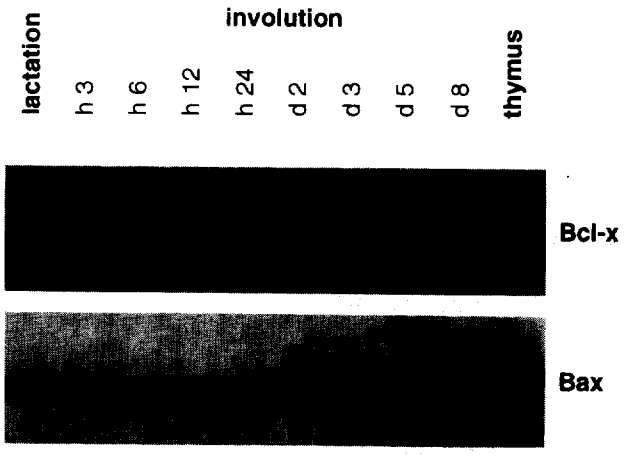

Fig. 4. Western blot analysis of bcl-x and bax during involution. Cell lysate from thymus was used as positive control.

mary gland involution. bcl-x_S and bax mRNA levels in mammary epithelial cells increase within the first day after weaning, coinciding with the induction of apoptosis. Increased amounts of Bax protein are found in apoptotic alveolar cells which are being shed from collapsing and remodeled alveoli. These results are a strong indication that both Bax and Bcl-x_S play an important role in the apoptosis of mammary alveolar cells during involution.

The p53 tumor-suppressor has been linked to apoptosis in cell culture. Ectopic expression of p53 targeted to mammary tissue of pregnant mice results in a massive loss of alveolar cells (Li et al., 1994), suggesting that this molecule has the ability to induce apoptosis in the context of the whole animal. The mechanism of induction may be the upregulation of bax transcription, as shown in transfection studies in murine leukemia and human lung cancer cells (Selvakumaran et al., 1994; Miyashita and Reed, 1995). However, the strong induction of bax mRNA during the first 3 days of mammary gland involution is unchanged in animals lacking p53. Furthermore, we have recently demonstrated that apoptosis during normal involution (Li et al., 1996a) and oncoprotein-induced apoptosis during pregnancy is independent of functional p53 (Li et al., 1996b). This strongly suggests that p53-independent apoptosis pathways are used in the mammary gland.

Members of the bcl-2 family of genes mediate apoptosis by forming homo- and heterodimers (Sedlak et al., 1995) and the relative levels of each protein in an individual cell may determine whether or not a cell will undergo apoptosis (Oltvai and Korsmeyer, 1994). Bcl-2, the prototype of the bcl-2 family, does not appear to play a dominant role in mammary gland involution.

Expression of the bcl-2 gene during lactation and involution was very low and constitutive (unpublished observation), and mammary development is unaltered in bcl-2 null mice (Veis et al., 1993). In contrast, bcl-x and bax, which are closely related to bcl-2, exhibit features compatible with a critical role in apoptosis during mammary tissue remodeling. We originally observed that elevated levels of these molecules coincided with oncoprotein-induced apoptosis of mammary alveolar cells in pregnant transgenic mice expressing the SV40 T-antigen under control of the WAP gene promoter (Li et al., 1996b). We now demonstrate that a transient induction of these proteins occurs also during alveolar apoptosis in the normal involution process following weaning. Bcl-x can be transcribed and processed into two different RNA forms with opposing functions (Boise et al., 1993). Bcl-x_L is the more abundant form and inhibits apoptosis. Bcl-x_S is expressed at lower levels and accelerates cell death in cell transfection studies. Because of its lower expression levels, the role of bcl-x_S in promoting apoptosis under physiological conditions has not been obvious. González-García and coworkers have analyzed bcl-x expression in several organs during embryonic development and in the adult organism (but not in mammary tissue) and did not detect bcl-x_S, arguing against a widespread role of bcl-x_S during development (González-García et al., 1994). In the mammary gland, however, bcl-x_S mRNA is present.

The expression pattern of the bcl-x and bax genes during lactation and involution, in conjunction with the increase of the bcl-x_S mRNA shortly after weaning, supports a role for these proteins in apoptosis during remodeling of mammary tissue. The expression pattern of Bcl-x protein followed closely the expression of the RNA. While bax mRNA was induced in early involution and decreased after day 3, Bax protein levels did not change significantly up to day 5 and increased thereafter. The disappearance of bax mRNA 3 days after weaning could be explained by RNA degradation in dying cells. The protein may be more stable, an assumption that is supported by the appearance of large cells in the alveolar lumina that stain heavily for Bax protein and may represent macrophages with engulfed cell debris. The reported half-life for Bax is cell type-dependent and can be longer than 24 h (Miyashita et al., 1996). In immunohistochemistry strong expression of Bax protein was seen in dying cells, clearly demonstrating a correlation between the presence of Bax and apoptosis. By biochemical and morphological criteria, the peak of cell death was observed 3 days after weaning. Cell culture studies have demonstrated that human bcl-x_S and bax expression sensitizes cells to cell death signals, probably by inhibiting the ability of bcl-2 to promote cell survival. Cells die within 24-48h (Boise et al., 1993; Oltvai et al., 1993). In mammary gland, the increase of steady state levels of bcl-x_S and bax mRNA preceded apoptosis by 24–48 h, thus temporally coinciding with the decision of alveolar cells to undergo apoptosis. Interestingly, even at the height of apoptosis the bcl-x_S mRNA seems to be less abundant

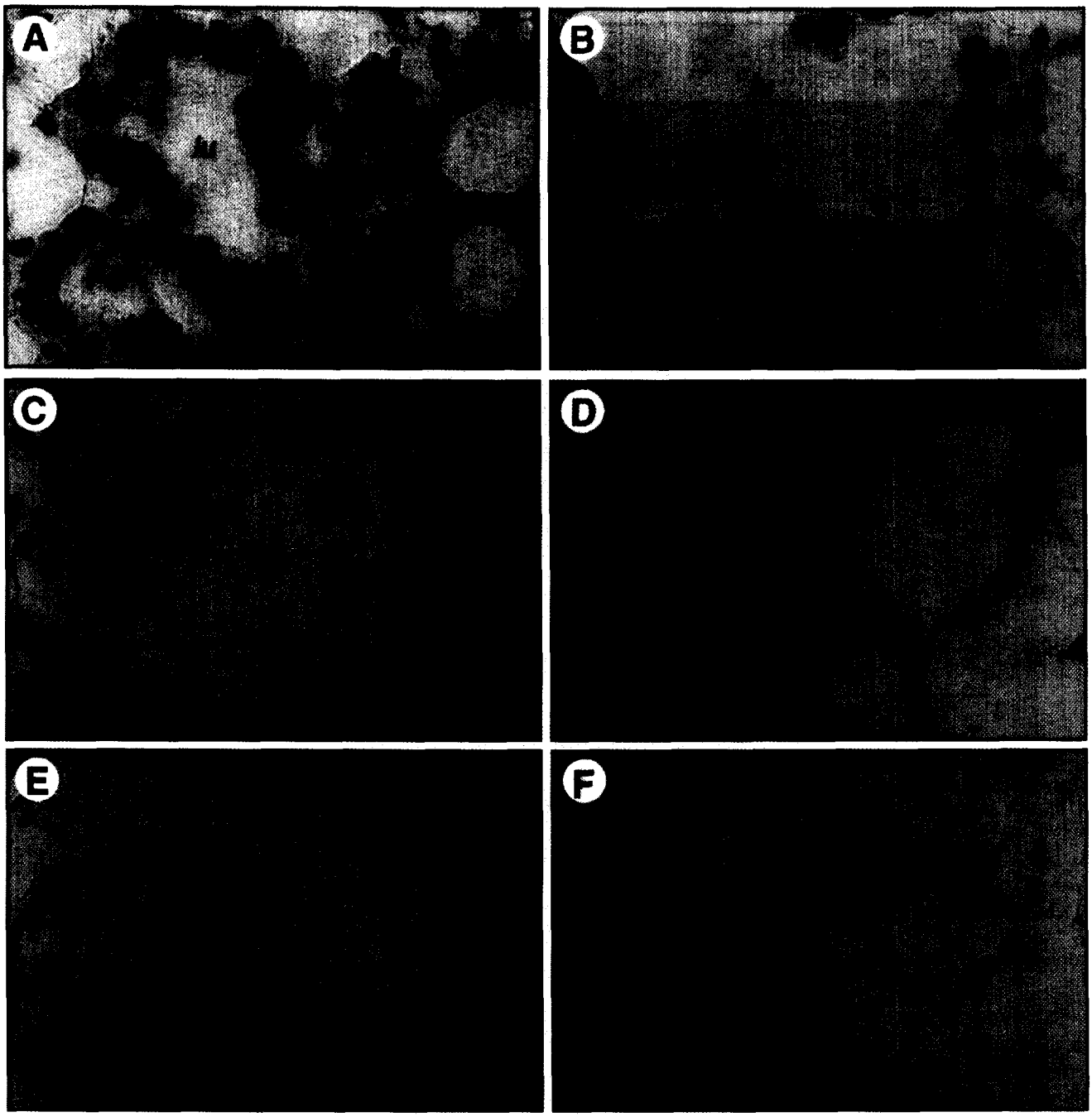

Fig. 5. Immunohistochemical localization of bax during lactation and involution. (A) Day 3 of lactation; (B) day 2 of involution; (C) day 3 of involution; (D,E) day 5 of involution; (F) day 8 of involution. lu, lumen. The arrow in (B) depicts an alveolar cell with a fragmented nucleus and strongly staining for bax, which is being shed into the lumen. The arrows in (C) point to bax-expressing cells located in collapsing alveoli. The arrows in (D) identify bax-positive cells which are still part of an alveolus. The open arrow in (E) points to a cell of unknown origin. (F) shows a gland that has been largely remodeled. Some bax-positive cells are still visible in collapsed alveoli. Bar is 25 μm.

than bcl-x_L mRNA. This is in agreement with our cell culture studies in which bcl-x_S can increase the apoptosis rate in cells in the presence of a three-fold excess of bcl-x_L. Similarly, bcl-x_S can facilitate apoptosis in a human breast cancer cell line expressing excess bcl-x_L (Sumantran et al., 1995). These experiments demonstrate that small changes in bcl-x_S levels can affect cell viability. The correlation between the number of apoptotic cells detected by histology and the ratio of bcl-x_S versus bcl-x_L suggests that the induction of bcl-x_S may occur in only a few cells which proceed into apoptosis. The ratio of molecules which function to repress cell death (Bcl-2 and Bcl-x_L) to those promoting apoptosis (Bax and Bcl-x_S) may represent a cell-autonomous rheostat that determines a cell's response to an apoptotic stimulus (Oltvai et al., 1993; Sato et al., 1994; Yang et al., 1995).

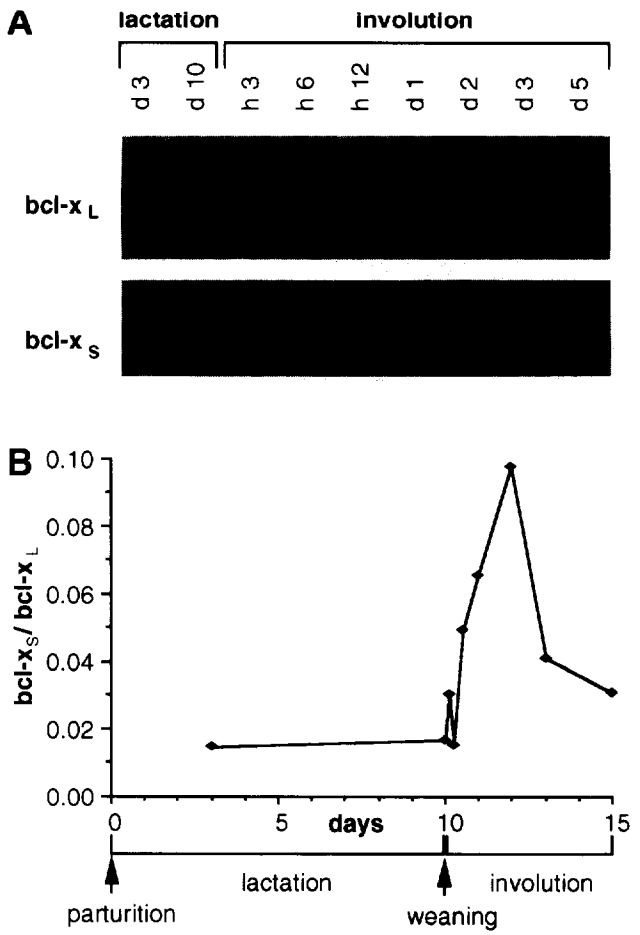

Fig. 6. Expression of bcl-x_S and bcl-x_L during lactation and involution. (A) RT-PCR products are hybridized with oligonucleotides specific for the short and long splice forms of bcl-x. (B) Ratio of bcl-x_S versus bcl-x_L.

4. Material and methods

4.1. Mice

C57BL/6 wildtype and 129 p53 null females were used for these studies. The mice that contained two defective germ-line p53 alleles carried a targeted disruption of the p53 gene (The Jackson Laboratory, Bar Harbor, ME) (Jacks et al., 1994).

4.2. Tunel assay

Mammary gland specimens were fixed in 4% paraformaldehyde in PBS and embedded in paraffin by standard methods. Sections (5 μm) were mounted on silan-coated slides. Apoptotic cell nuclei were identified using the ApopTag kit (Oncor, Gaithersburg, MD). Sections were initially treated with 20 μg/ml proteinase K in PBS for 15 min at room temperature, quenched by 0.0003% H_2O_2 for 30 min at room temperature, equilibrated with buffer, incubated with TdT for 20–40 min at room temperature, washed with wash stop buffer for 30 min at 37°C, and incubated with anti-digoxigenin for 30 min at room temperature. Color was developed using 0.05% 3,3′dimethylaminoazobenzene, 0.01% H_2O_2 diluted in 0.1 M Tris–HCl (pH 7.5) and counterstained with methyl green.

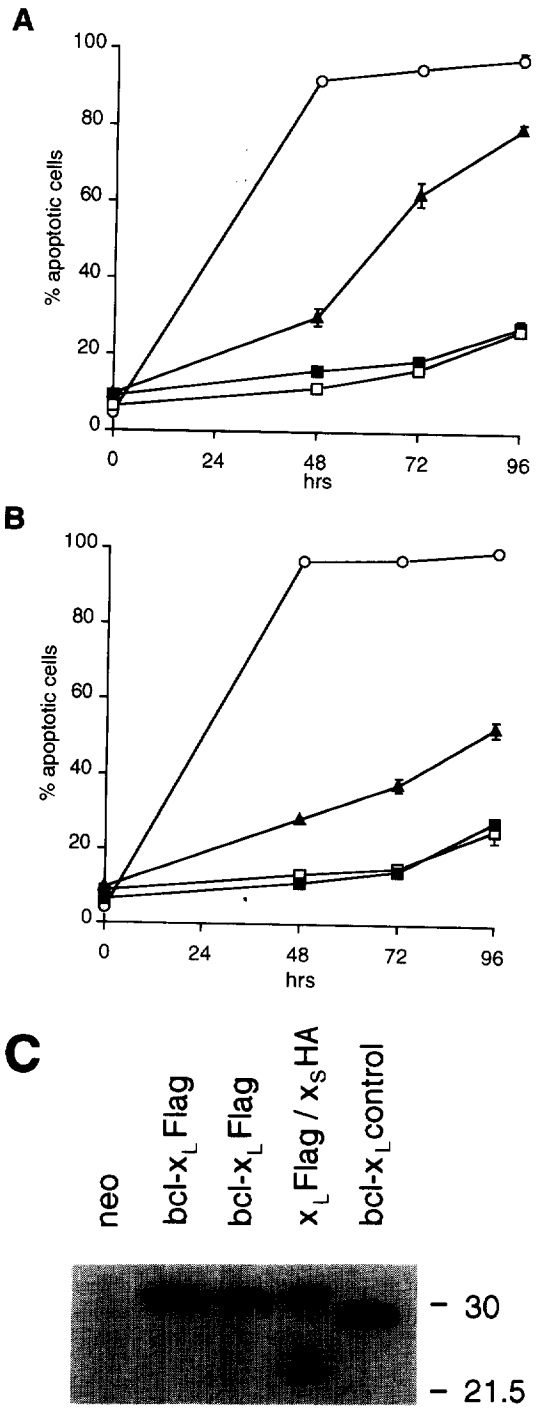

Fig. 7. (A,B) Apoptosis assay of FL5.12 cells transfected with vectors expressing neo (open circles) or bcl-x_L Flag (open and filled squares, representing two independent clones) or cotransfected with bcl-x_L Flag and bcl-x_S HA (filled triangles). Standard deviation is indicated by the error bars. (C) Western blot analysis of bcl-x_L and bcl-x_S expression in the transfected cell lines. FL5.12 cells transfected with a vector expressing bcl-x_L without epitope tag was used as positive control.

4.3. Northern blot analysis

RNA was prepared according to Chomczynski and Sacchi (1987). Electrophoresis of RNA was performed using the formaldehyde method (Sambrook et al., 1989); 20 μg of total RNA were used per lane. The RNA was transferred to nylon membranes (GeneScreen Plus). To estimate the amount of RNA on the membrane, RNA was stained with methylene blue (Herrin and Schmidt, 1988). The filters were hybridized with probes described below and washed using standard protocols.

4.4. In situ hybridization

Mammary gland tissue was embedded in Miles embedding medium and frozen on dry ice. Sections (12–16 μm) were mounted on silan-coated slides and used for in situ hybridization. Sections were fixed in 4% paraformaldehyde in PBS, treated with 2 μg/ml proteinase K for 10 min at room temperature, post-fixed in 4% paraformaldehyde and acetylated. After prehybridization in 0.3 M NaCl, 20 mM Na-acetate (pH 5), 5 mM EDTA, 50% formamide, 10% Dextran sulfate, 1 × Denhardt's solution, 100 mM DTT, plus 80 μg/μl yeast tRNA for 2–3 h, ^{35}S-labeled cRNA probes were hybridized overnight at 45°C in the same solution using ca. 3 × 10^7 cpm/ml. Sections were washed in 4 × SSC, 10 mM DTT, at RT (4 × 15 min) and treated with 20 μg/ml RNaseA in 0.5 M NaCl, 10 mM Tris–HCl (pH 8), 1 mM EDTA, at 37°C for 30 min. Further washes were performed in 0.5 M NaCl, 10 mM Tris–HCl (pH 8), 1 mM EDTA, 10 mM β-mercaptoethanol at 37°C (4 × 10 min), 2 × SSC, 10 mM β-mercaptoethanol at 37°C (4 × 15 min), and 15 min in 0.1 × SSC, 10 mM β-mercaptoethanol at 50°C. Sections were dried and dipped in Kodak NTB-2 emulsion, 600 mM NH_4-acetate, exposed at 40°C for 1 week and developed in Kodak D-19.

4.5. Probes for hybridization

To generate a probe specific for mouse bcl-x, we used partially degenerate primers from conserved stretches of human and chicken cDNA. PCR amplification of mouse genomic DNA with the primer pair ACCGGGAGc/tTa/gGTGa/gTTGACTTT and GGGGTGATGTGGAGCTGGGA resulted in a DNA fragment of 334 bp. RT-PCR was performed to generate a mouse bax probe from total RNA of a mammary gland 1 day after weaning. The primer pair CTTGGAGCAGCCGCCCCAGG and GGCACTTTAGTGCACAGGGC amplified a fragment of 260 bp. Both fragments were cloned into the vector pCR™II (Invitrogen). Sequencing proved that the clones were identical to the corresponding regions of the published bcl-x (González-García et al., 1994) and bax mouse sequences (Oltvai and Korsmeyer, 1994) For Northern hybridization the inserts were isolated and labeled with ^{32}P by random prime labeling (Stratagen). For in situ hybridization ^{35}S-labeled RNA probes were generated by transcription of linearized plasmids (Boehringer).

4.6. RT PCR and DNA hybridization

For RT-PCR 1 μg total RNA was reverse transcribed in 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 125 μM each ATP, CTP, GTP, and TTP, 10 mM DTT, 2 μM specific primer (CATGCCCGTCAGGAACCAGCGG), reading upstream, and 200 U MLV RT (Gibco). Half of the RT product was used in a 50 μl PCR reaction. Using an annealing temperature of 65°C, 25 cycles of PCR were performed in 25 mM Tris–HCl (pH 8.3), 40 mM KCl, 2 mM $MgCl_2$, 50 μM each ATP, CTP, GTP, and TTP, 0.01% gelatin, 0.4 μM both specific primers (downstream primer: GCGCGGGAGGTGATTCCCATGGC), and 1.5 U Taq polymerase (Promega). PCR products were separated on a 1.2% agarose gel, denatured and blotted on nylon membranes (GeneScreen Plus). The UV crosslinked DNA was hybridized to [α^{32}P] end-labeled specific oligonucleotides (CGCTTTCCACGCACAGTGCCCCGCC for bcl-x_L and CAGAGCTTTGAGCAGGACACTTTTGTGG for bcl-x_S, spanning the region of the splice site) at 60°C in 0.4 M NaCl, 1% SDS, 5 × Denhardt's solution, and 100 μg/ml salmon sperm DNA for 12–18 h. Membranes were washed in 2 × SSC, 0.1% SDS at 60°C and 65°C. The resulting signals were quantitated using a phosphoImager (Molecular Dynamics).

4.7. Western blot analysis

Total cell lysate was prepared from mammary tissue and thymus using RIPA (1% NP40, 0.5% sodium deoxycholate, 0.1% SDS in PBS). Proteinase inhibitors PMSF, aprotinin and sodium orthovanadate were added. Protein (50 μg) was fractionated in a SDS-14% polyacrylamide gel and transferred to PVDF membranes (Novex). After blocking in 5% non-fat milk in T-PBS (0.1% Tween 20, 10 mM Tris–HCl (pH 7.5), 0.1 M NaCl) for 1 h at room temperature, the membranes were incubated in 0.5 μg/ml anti-bax antibody (N20) or anti-bcl-x antibody (S18) (Santa Cruz Biotechnology) in blocking buffer for 1 h at room temperature, washed in T-PBS, and incubated in a 1:4000 dilution of peroxidase-conjugated goat anti-rabbit IgG polyclonal antibody (Sigma Immunochemicals) for 1 h at room temperature. Total lysates from FL5.12 cells were prepared by lysing cells in 1% SDS/PBS. Lysates were boiled for 10 min, sonicated, and microfuged to remove debris. Lysates from 2 × 10^6 cells were boiled in 2 × sample buffer, run on a 14% polyacrylamide/SDS gel, and transferred onto a 0.2 μm PVDF membrane. The blot was incubated with a 1:5000 dilution of a monoclonal anti-human Bcl-x antibody (courtesy of Dr. Craig Thompson), washed, and incubated with a 1:50 000 dilu-

tion of goat anti-mouse IgG Fc-HRP antibody (Jackson Immunoresearch). The ECL Western blotting kit (Amersham) was used for detection. As a loading control, filters were stained with Poncean stain (Daitbother) or gels were stained with Coomassie blue (Novex).

4.8. Immunohistochemistry

Paraffin sections were deparaffinized, treated with 2 μg/ml of proteinase K in PBS for 10 min, and quenched in 0.3% H_2O_2 in PBS for 15 min. Pretreatment with 10% goat serum, and 0.1% BSA in PBS was followed by an incubation in 0.5 μg/ml anti-bax antibody in the same buffer for 1 h at room temperature. The Vectastain Elite ABC kit (Vector) was used for detection according to manufacturers protocol. The color reaction was performed in Sigma Fast DAB reagent (Sigma).

4.9. Cell culture

The murine IL-3-dependent pro B-cell line FL5.12 was maintained in RPMI (Gibco, Grand Island, NY) supplemented with 10% FCS, and 10% WEHI-3B (D-)-conditional medium as a source of IL-3 as described (Nuñez et al., 1990).

4.10. Transfection of cells

An eight amino-acid Flag epitope tag (Hopp et al., 1988) and a ten amino-acid HA epitope (Kolodziej and Young, 1991) were attached by PCR to the N-termini of human Bcl-x_L or Bcl-x_S, respectively.

The FL5.12 murine pre-B cell line was singly transfected with 20 μg of pSFFVneo Bcl-x_L Flag or pSFFVneo Bcl-x_S HA, or was co-transfected with 10 μg of each plasmid by electroporation (200 V, 960 μF). Cells were plated at 5000 cells/well, and stably transfected clones were selected for neomycin resistance by growth in 2 mg/ml G418 (Gibco). Surviving clones were screened for expression of either Bcl-x_L Flag, Bcl-x_S HA or both by Western blotting and Flow cytometric analysis using the Flag M2 antibody (IBI) or anti-HA monoclonal 12CA5 (Boehringer Mannheim). Single Bcl-x_L Flag, single Bcl-x_S HA, and double expressing clones were selected for further analysis. Apoptosis in cells treated with etoposide or IL-3 withdrawal was measured by propidium iodide staining as previously described (Grillot et al., 1995).

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